PROVA ESTRATTA

Prova 3

E' stata richiesta l'esecuzione di un test per l'accertamento della paternità biologica.

Mediante kit commerciale l'AmpF/STR® Identifiler™ PCR Amplification Kit (Applied Biosystem) sono stati tipizzati tre campioni di DNA che appartengono a: M: madre; F: figlio; PP: presunto padre.

I risultati complessivamente ottenuti sono riportati nella sottostante tabella:

locus	**PP**	**F**	**M**
D8S1179	13/15	13/13	12/13
D21S11	29/31.2	29/32.2	29/29
D7S820	11/11	8/12	8/8
CSF1PO	10/11	11/12	10/11
D3S1358	16/18	16/16	14/16
TH01	7/9	9/9.3	9.3/9.3
D13S317	9/11	8/8	8/11
D16S539	11/13	9/11	9/9
D2S1338	17/17	19/23	23/23
D19S433	14/14	14/14	14/15.2
VWA	15/17	18/18	17/18
TPOX	10/10	10/11	8/11
D18S51	13/16	12/13	13/19
D5S818	10/12	11/12	12/12
FGA	20/24	22/23	22/27
Amel.	X/Y	X/Y	X/X

Le caratteristiche del kit AmpF/STR® Identifiler™ PCR Amplification Kit sono riportate in **allegato 1**.

Spiegare i risultati e pianificare la strategia analitica.

PS: all'**allegato 2** si trovano le frequenze alleliche italiane di riferimento.

Product overview

Purpose

The AmpFℓSTR® Identifiler® Plus PCR Amplification Kit is a short tandem repeat (STR) multiplex assay that amplifies 15 tetranucleotide repeat loci and the Amelogenin gender-determining marker in a single PCR amplification:

- All thirteen of the required loci for the Combined DNA Index System (CODIS) loci are included in this kit for known-offender databasing in the United States (Budowle *et al.*, 1998).
- Two additional loci, D2S1338 and D19S433, are included. These loci are consistent with the AmpFℓSTR SGM Plus™ PCR Amplification Kit.
- The combination of the 15 loci are consistent with several worldwide database recommendations.

The AmpFℓSTR Identifiler Plus Kit delivers a 16-locus multiplex with the same power of discrimination as, better sensitivity than, and better robustness than the earlier generation of the AmpFℓSTR Identifiler Kit. The kit uses modified PCR cycling conditions for enhanced sensitivity, a new buffer formulation to improve performance with inhibited samples, and an improved process for DNA synthesis and purification of the amplification primers to deliver a much cleaner electrophoretic background.

Product description

The AmpFℓSTR Identifiler Plus Kit uses the same primer sequences as the earlier generation AmpFℓSTR® Identifiler® Kit. The AmpFℓSTR Identifiler Plus Kit contains all the necessary reagents for the amplification of human genomic DNA.

The reagents are designed for use with the following Applied Biosystems instruments:

- ABI PRISM® 3100/3100-*Avant* Genetic Analyzer
- Applied Biosystems 3130/3130*xl* Genetic Analyzer
- Applied Biosystems 310 Genetic Analyzer
- GeneAmp® PCR System 9700 with the Silver 96-Well Block
- GeneAmp® PCR System 9700 with the Gold-plated Silver 96-Well Block
- Veriti® 96-Well Thermal Cycler

About the primers

The AmpFℓSTR Identifiler Plus Kit employs the latest improvements in primer synthesis and purification techniques to minimize the presence of dye-labeled artifacts. These improvements result in a much cleaner electropherogram background that enhances the assay's signal-to-noise ratio and simplifies the interpretation of results.

Non-nucleotide linkers are used in primer synthesis for the following loci: CSF1PO, D13S317, D16S539, D2S1338, and TPOX. For these primers, non-nucleotide linkers are placed between the primers and the fluorescent dye during oligonucleotide synthesis (Butler, 2005, Grossman *et al.*, 1994, and Baron *et al.*, 1996). Non-nucleotide linkers enable reproducible positioning of the alleles to facilitate

interlocus spacing. The combination of a five-dye fluorescent system and the inclusion of non-nucleotide linkers allows for simultaneous amplification and efficient separation of the 15 STR loci and Amelogenin during automated DNA fragment analysis.

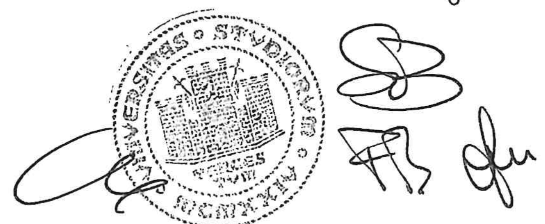
Loci amplified by the kit

The following table shows the loci amplified, their chromosomal locations, and the corresponding fluorescent marker dyes. The AmpFℓSTR® Identifiler® Plus Allelic Ladder is used to genotype the analyzed samples. The alleles contained in the allelic ladder, and the genotype of the AmpFℓSTR® Identifiler® Plus Control DNA 9947A are also listed in the table.

Table 1 AmpFℓSTR® Identifiler® Plus Kit loci and alleles

Locus designation	Chromosome location	Alleles included in Identifiler® Plus Allelic Ladder	Dye label	Control DNA 9947A
D8S1179	8	8, 9 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	6-FAM™	13‡
D21S11	21q11.2-q21	24, 24.2, 25, 26, 27, 28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36, 37, 38		30§
D7S820	7q11.21-22	6, 7, 8, 9, 10, 11, 12, 13, 14, 15		10, 11
CSF1PO	5q33.3-34	6, 7, 8, 9, 10, 11, 12, 13, 14, 15		10, 12
D3S1358	3p	12, 13, 14, 15, 16, 17, 18, 19	VIC®	14, 15
TH01	11p15.5	4, 5, 6, 7, 8, 9, 9.3, 10, 11, 13.3		8, 9.3
D13S317	13q22-31	8, 9, 10, 11, 12, 13, 14, 15		11#
D16S539	16q24-qter	5, 8, 9, 10, 11, 12,13, 14, 15		11, 12
D2S1338	2q35-37.1	15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28		19, 23
D19S433	19q12-13.1	9, 10, 11, 12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2	NED™	14, 15
vWA	12p12-pter	11,12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24		17, 18
TPOX	2p23-2per	6, 7, 8, 9, 10, 11, 12, 13		8‡‡
D18S51	18q21.3	7, 9, 10, 10.2, 11, 12, 13, 13.2, 14, 14.2, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27		15, 19
Amelogenin	X: p22.1-22.3 Y: p11.2	X, Y	PET®	X
D5S818	5q21-31	7, 8, 9, 10, 11, 12, 13, 14, 15, 16		11§§
FGA	4q28	17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 26.2, 27, 28, 29, 30, 30.2, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 50.2, 51.2		23, 24

‡ For CODIS purposes, profile reported as 13, 13.
§ For CODIS purposes, profile reported as 30, 30.
# For CODIS purposes, profile reported as 11, 11.
‡‡ For CODIS purposes, profile reported as 8, 8.
§§ For CODIS purposes, profile reported as 11, 11.



Allelic ladder profile

Figure 1 shows the allelic ladder for the AmpFℓSTR Identifiler Plus Kit. See "Allelic ladder requirements" on page 27 for information on ensuring accurate genotyping.

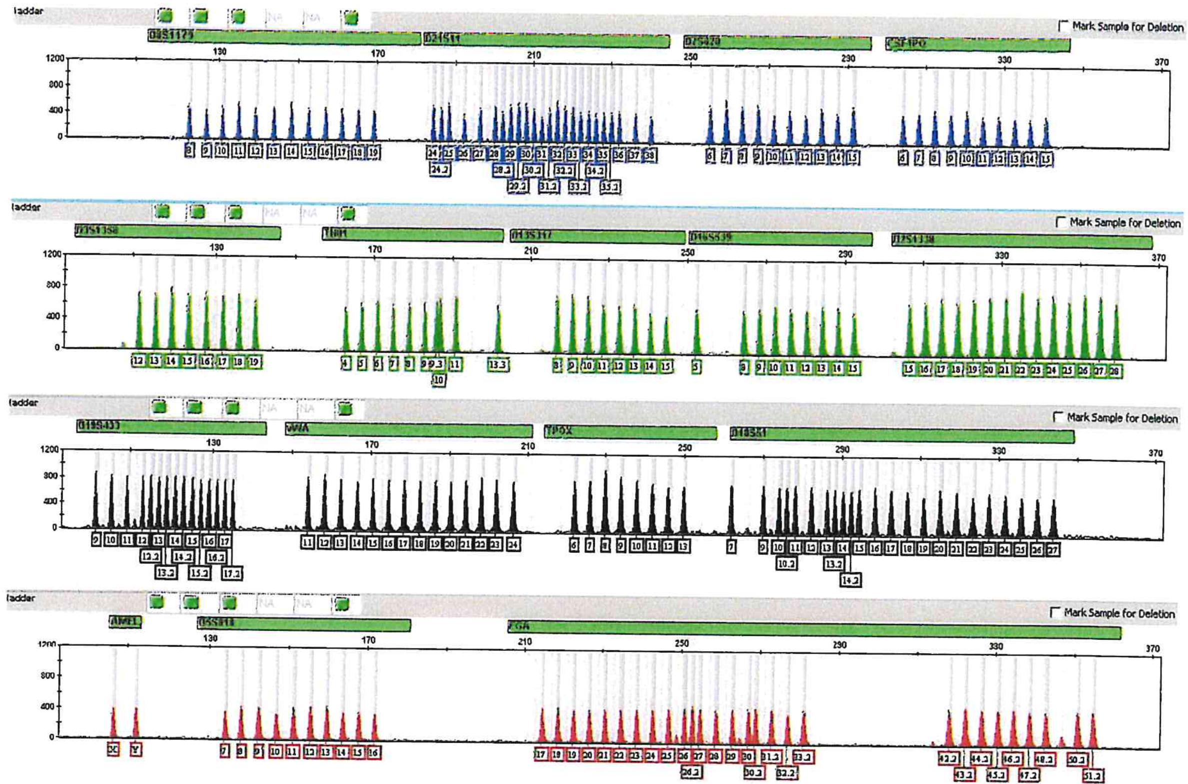


Figure 1 GeneMapper® *ID-X* Software plot of the AmpFℓSTR® Identifiler® Plus Kit Allelic Ladder

Control DNA 9947A profile

Figure 2 shows amplification of Control DNA 9947A using the AmpFℓSTR Identifiler Plus Kit.

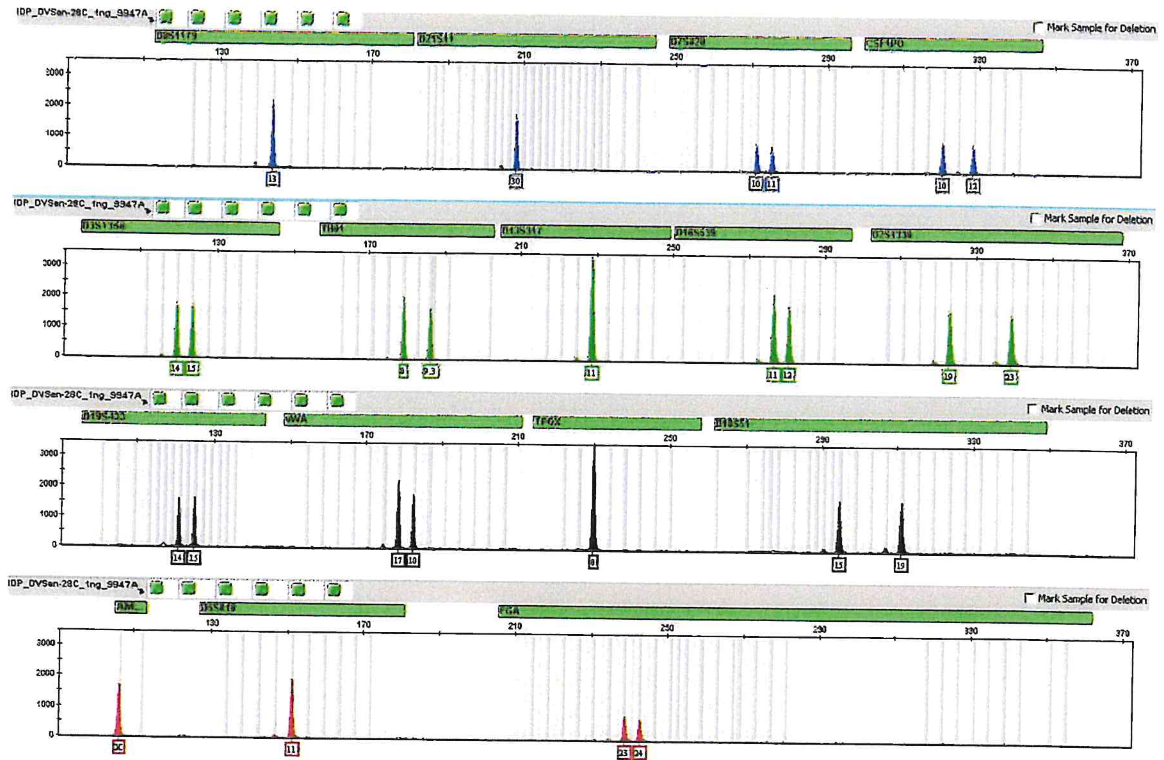
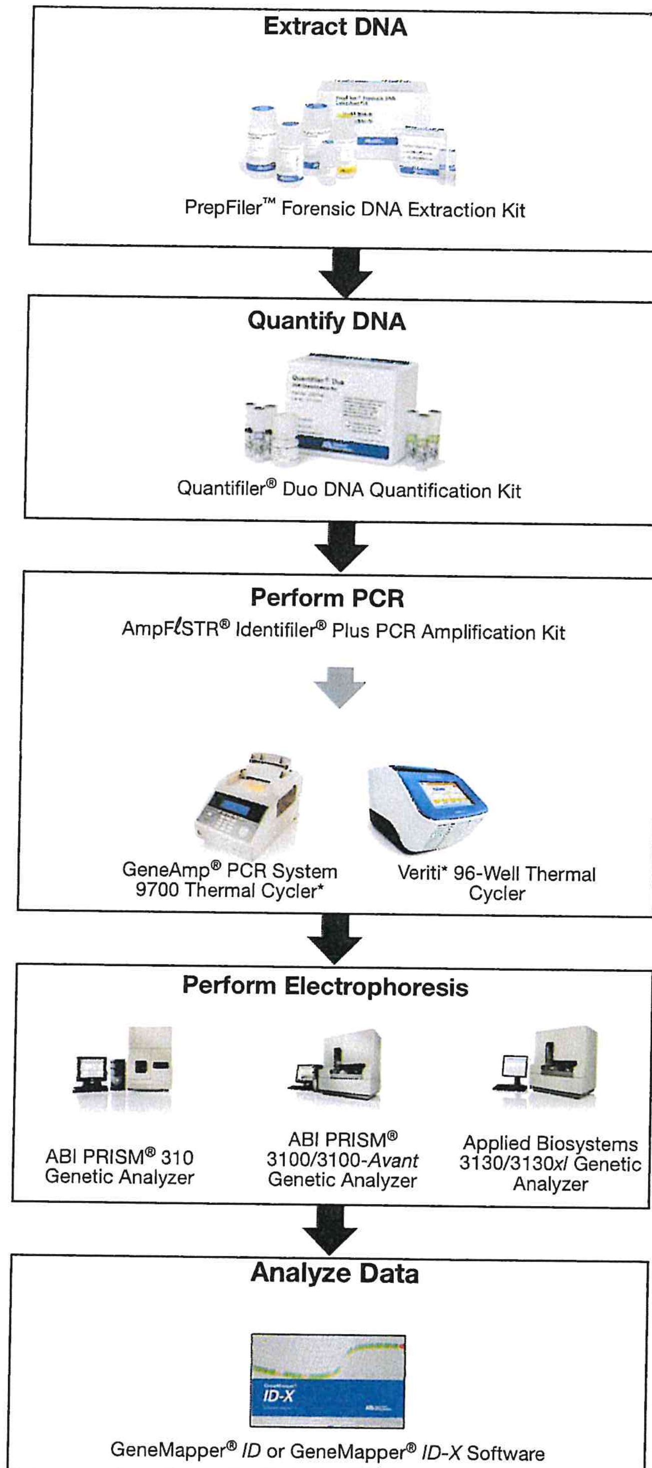
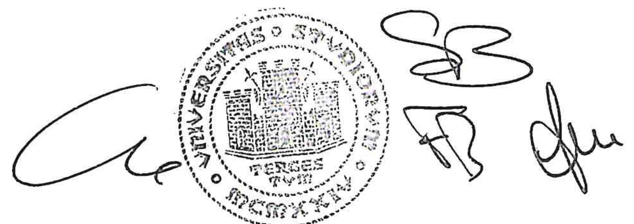


Figure 2 1 ng of Control DNA 9947A amplified with the AmpFℓSTR® Identifiler® Plus Kit and analyzed on the Applied Biosystems 3130*xl* Genetic Analyzer

Workflow overview





Instrument and software overview

This section provides information about the Data Collection Software versions required to run the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit on specific instruments.

Data Collection and GeneMapper® *ID* or *ID-X* Software

The Data Collection Software provides instructions to firmware running on the instrument and displays instrument status and raw data in real time. As the instrument measures sample fluorescence with its detection system, the Data Collection Software collects the data and stores it. The Data Collection Software stores information about each sample in a sample file (.fsa), which is then analyzed by the GeneMapper® *ID* or *ID-X* Software.

Instrument and software compatibility

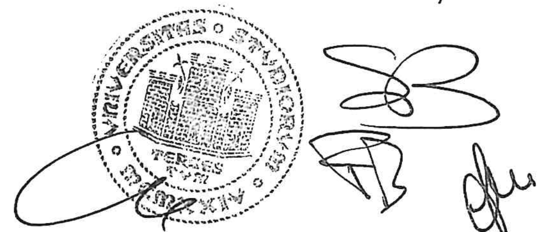
Instrument	Operating system	Data Collection Software	Analysis software
3130/3130*xl*‡	Windows® XP	3.0	• GeneMapper® *ID* Software v3.2.1 *and* • GeneMapper® *ID-X* Software v1.0.1 or higher
3100/3100-*Avant*	Windows NT®	1.1 (3100) 1.0 (3100-*Avant*)	
	Windows 2000	2.0	
310	Windows XP	3.1	
	Window NT and Windows 2000	3.0	

‡ Applied Biosystems conducted validation studies for the AmpFℓSTR® Identifiler® Plus Kit using this configuration.

About multicomponent analysis

Applied Biosystems fluorescent multi-color dye technology allows the analysis of multiple loci, including loci that have alleles with overlapping size ranges. Alleles for overlapping loci are distinguished by labeling locus-specific primers with different colored dyes.

Multicomponent analysis is the process that separates the five different fluorescent dye colors into distinct spectral components. The four dyes used in the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit to label samples are 6-FAM™, VIC®, NED™, and PET® dyes. The fifth dye, LIZ® dye, is used to label the GeneScan™ 500 LIZ® Size Standard.



How multicomponent analysis works

Each of these fluorescent dyes emits its maximum fluorescence at a different wavelength. During data collection on the Applied Biosystems and ABI PRISM® instruments, the fluorescence signals are separated by diffraction grating according to their wavelengths and projected onto a charge-coupled device (CCD) camera in a predictably spaced pattern. The 6-FAM™ dye emits at the shortest wavelength and it is displayed as blue, followed by the VIC® dye (green), NED™ dye (yellow), PET® dye (red), and LIZ® dye (orange).

Although each of these dyes emits its maximum fluorescence at a different wavelength, there is some overlap in the emission spectra between the dyes (Figure 3). The goal of multicomponent analysis is to correct for spectral overlap.

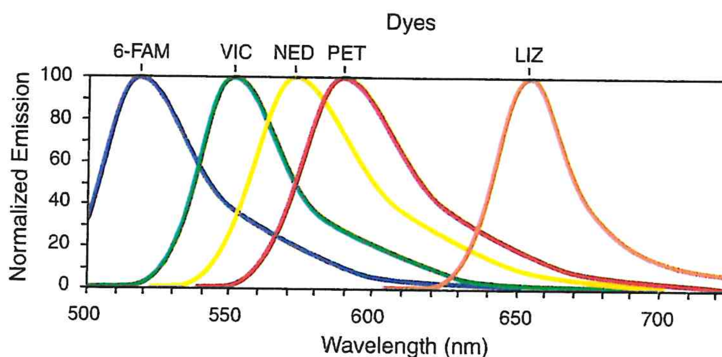


Figure 3 Emission spectra of the five dyes used in the AmpFℓSTR® Identifiler® Plus Kit

Materials and equipment

Kit contents and storage

The AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (PN 4427368) contains materials sufficient to perform 200 amplifications at 25 μL reaction volumes.

IMPORTANT! The fluorescent dyes attached to the primers are light sensitive. Protect the primer set from light when not in use. Amplified DNA, AmpFℓSTR® Identifiler® Plus Allelic Ladder, and GeneScan™ 500 LIZ® Size Standard should also be protected from light. Keep freeze-thaw cycles to a minimum.

Component	Description	200× Volume	Storage
AmpFℓSTR® Identifiler® Plus Primer Set	Contains forward and reverse primers to amplify human DNA targets.	1 tube, 1.0 mL	-15 to -25 °C on receipt, 2 to 8 °C after initial use
AmpFℓSTR® Identifiler® Plus Master Mix	Contains enzyme, salts, dNTPs, carrier protein, and 0.04% sodium azide.	2 tubes, 1.0 mL each	-15 to -25 °C on receipt, 2 to 8 °C after initial use
AmpFℓSTR® Identifiler® Plus Allelic Ladder	Contains amplified alleles. See Table 1 on page 3 for a list of alleles included in the allelic ladder.	1 tube, 50.0 μL	-15 to -25 °C on receipt, 2 to 8 °C after initial use
AmpFℓSTR® Control DNA 9947A	Contains 0.10 ng/μL human female 9947A DNA in 0.05% sodium azide and buffer‡. See Table 1 on page 3 for profile.	1 tube, 0.3 mL	2 to 8 °C

‡ The AmpFℓSTR® Control DNA 9947A is included at a concentration appropriate to its intended use as an amplification control (i.e., to provide confirmation of the capability of the kit reagents to generate a profile of expected genotype). The AmpFℓSTR® Control DNA 9947A is not designed to be used as a DNA quantitation control, and laboratories may expect to see variation from the labelled concentration when quantitating aliquots of the AmpFℓSTR® Control DNA 9947A.

Standards for samples

For the AmpFℓSTR Identifiler Plus Kit, the panel of standards needed for PCR amplification, PCR product sizing, and genotyping are:

- **Control DNA 9947A** – A positive control for evaluating the efficiency of the amplification step and STR genotyping using the AmpFℓSTR® Identifiler® Plus Allelic Ladder.
- **GeneScan™ 500 LIZ® Size Standard** – Standard used for obtaining sizing results. It contains 16 single-stranded labeled fragments of: 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490 and 500 nucleotides. This standard, which has been evaluated as an internal lane size standard, yields precise sizing results for AmpFℓSTR Identifiler Plus Kit PCR products. Order the GeneScan 500 LIZ Size Standard (PN 4322682) separately.
- **AmpFℓSTR® Identifiler® Plus Allelic Ladder** – Allelic ladder developed by Applied Biosystems for accurate characterization of the alleles amplified by the AmpFℓSTR Identifiler Plus Kit. The AmpFℓSTR® Identifiler® Plus Allelic Ladder contains most of the alleles reported for the 15 autosomal loci. Refer to Table 1 on page 3 for a list of the alleles included in the AmpFℓSTR® Identifiler® Plus Allelic Ladder.

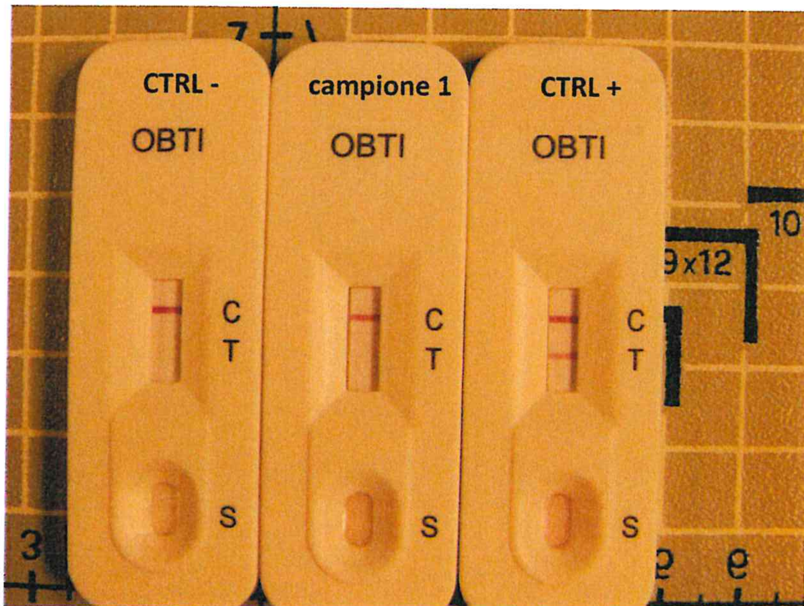
Alleles	TH01	VWA	FGA	D21S11	D3S1358	D8S1179	D18S51	D12S391	D2S441	D10S1248	D22S1045	D1S1656	D16S539	D2S1338	D19S433	SE33	TPOX	CSF1PO	D5S818	D13S317	D7S820	PentaD	PentaE
2.2																						0.0029	
3.2																							
4																							
5	0.0011																						0.0648
6	0.2358																0.0014				0.0014	0.0057	
7	0.1902																	0.0014			0.0129	0.0087	0.1519
8	0.0946					0.0174							0.0196				0.533	0.0029		0.1218	0.1648	0.0167	0.0248
9	0.1326					0.0054			0.0022				0.1033				0.116	0.0172	0.0415	0.0774	0.1762	0.1897	0.0138
9.1									0.0011														
9.3	0.3348																						
10	0.0098					0.0989	0.0076		0.213			0.0022	0.0511		0.0011		0.043	0.2421	0.0544	0.0444	0.2722	0.1287	0.0788
10.3																							
11	0.0011				0.0011	0.0674	0.0087		0.3337	0.0022	0.138	0.0815	0.313		0.0043	0.0011	0.2593	0.3181	0.3927	0.2981	0.1805	0.1897	0.1208
11.3									0.0598														
12		0.0022			0.0011	0.163	0.1185		0.0457	0.0304	0.012	0.1098	0.313		0.0696	0.0054	0.0473	0.328	0.3524	0.308	0.1476	0.1577	0.1829
12.2															0.0011								
12.3									0.0054														
13		0.0011			0.0011	0.3468	0.125		0.0293	0.2913	0.0054	0.0663	0.1696		0.2587	0.0152		0.0731	0.1547	0.1117	0.0372	0.2178	0.1178
13.2															0.0098								
14		0.0935			0.1152	0.1674	0.1391		0.2522	0.3076	0.0522	0.0783	0.0304		0.3598	0.025		0.0143	0.0014	0.0372	0.0072	0.0567	0.0478
14.2							0.0011								0.0239	0.0043							
14.3												0.0033											
15		0.0924			0.2727	0.1011	0.1739	0.0348	0.0554	0.1957	0.3207	0.149		0.0011	0.1554	0.0391		0.0029	0.0029	0.0014		0.0257	0.0478
15.2															0.0359								
15.3												0.0598											
16		0.2043			0.2174	0.0315	0.1424	0.0228	0.0022	0.1391	0.388	0.1467		0.037	0.0565	0.0402							0.0478
16.2							0.0022								0.0163	0.0011							
16.3												0.0554											
17		0.2956			0.2185	0.0011	0.1424	0.125		0.0304	0.0772	0.0478		0.1988	0.0054	0.0623							0.0338
17.2															0.0011								
17.3								0.0185				0.1293				0.0022							
18		0.1989	0.0239		0.1533		0.0707	0.1739		0.0011	0.0065	0.0054		0.0739		0.0717							0.0338
18.2															0.0011								
18.3								0.0239				0.0511				0.0011							
19		0.1022	0.0543		0.0185		0.038	0.1239		0.0022		0.0011		0.1304		0.0663							0.0168
19.2																0.0043							
19.3								0.0054				0.013											
20		0.0087	0.1196		0.0011		0.0141	0.1109						0.1565		0.0543							0.0138
20.2			0.0011													0.0109							
20.3																							
21		0.0011	0.1837				0.0098	0.1261						0.037		0.0239							0.0028
21.2			0.0054													0.0239							
22			0.1989				0.0054	0.1						0.0359		0.0152							
22.2			0.0109													0.038							
23			0.1511					0.0674						0.1011		0.0022							
23.2			0.0022													0.0435							
24			0.1402					0.0489						0.1076		0.0011							
24.2			0.0011													0.0217							
25			0.0772				0.0011	0.0152						0.0924									
25.2				0.0011												0.0402							
26			0.025					0.0022						0.0283									
26.2																0.0435							
27			0.0054	0.025				0.0011															
27.2																0.0946							
28				0.1576																			
28.2																0.0739							
28.3																0.0011							
29				0.1957																			
29.2				0.0022												0.063							
30				0.2794																			
30.2				0.0315												0.0576							
31				0.0641												0.0022							
31.2				0.1022												0.0217							
32				0.0076												0.0011							
32.2				0.0967												0.013							
33				0.0011												0.0011							
33.2				0.0293												0.0054							
34																0.0065							
34.2				0.0043												0.0011							
35				0.0011																			
36				0.0011																			

Prova 1

L'imbrattamento rossastro (campione 1) della sottostante figura è stato rinvenuto su un paio di jeans in sequestro da 5 anni.

Il Combur test (test speditivo per la diagnosi di sangue) ha fornito risultato positivo, come indicato dalla freccia bianca:

Quale test di conferma è stato eseguito il test OBTI, fornendo i risultati che si vedono nella foto sottostante (CTRL – e CTRL + indicano il controllo negativo e, rispettivamente, positivo):



Le caratteristiche del kit OBTI sono nell'**allegato 1**.

Fornire le possibili spiegazioni e descrivere la strategia analitica.

HEXAGON OBTI

Test Rapido immunocromatografico

per rilevare la presenza di tracce di sangue umano in un campione di feci

Human
Diagnostics Worldwide

Contenuti:

REF **HU-28009** 24 dispositivi per test

24 tubi di raccolta

Per uso forense **Principio dell'Esame**

Il campione da analizzare con presunta traccia di sangue umano viene trasferito in un tubo con mezzo di trasporto. Questa mistura viene aggiunta goccia a goccia al test. L'emoglobina umana (hHb) nel campione reagisce con il reagente caratterizzato da particelle di colore rosso e anticorpi monoclonali umani Hb. L'immunocomplesso migra nella zona del test dove viene catturato da un secondo anticorpo immobilizzato e diretto contro l'hHb formando una linea rossa (T) per indicare il risultato positivo. I reagenti non reattivi migrano ulteriormente, legandosi in una seconda linea dagli anticorpi anti-mouse IgG immobilizzati. Questa linea di controllo (C) indica il corretto funzionamento e utilizzo del test.

Reagenti

24 dispositivi per il Test, consistenti in un coniugato di particelle rosse e anticorpi (anti-hHb,mouse), anticorpi anti-hHb (mouse) immobilizzati, e anticorpi anti-mouse IgG (goat) individualmente sigillati con disseccante.
24 tubi di raccolta con TRIS Buffer, ph 7.6, 1 applicatore a stick

Conservazione e Stabilità

Il Kit Hexagon OBTI è stabile fino alla data di scadenza riportata solo quando viene conservato tra i **2 e i 25 °C**. Non va utilizzato oltre la data di scadenza. Evitare cicli di congelamento ed esposizione a temperatura> 30°C .

Caratteristiche Funzionali

Il test è in grado di rilevare minime quantità di emoglobina umana : *12.5ug* Hb in un campione fecale.
Un risultato positivo è solitamente rilevato in 3-5 minuti, ma un risultato negativo invece non va confermato prima del termine di 10 minuti.
Il kit Hexagon OBTI è specifico per i seguenti sottotipi/sottoinsiemi di emoglobina umana HbA1, HbA2, HbF, HbS.

Non è stata osservata cross-reattività dal sangue delle seguenti specie:			
Pecora	Mucca	Maiale	Cavallo
Capra	Coniglio	Tacchino	Oca
Pollo	Piccione	Cervo rosso	Cervo Sika
Tasso	Donnola	Ratto	Maiale giuneo
Aringa	Trota di Mare	Rospo	Elefante Africano

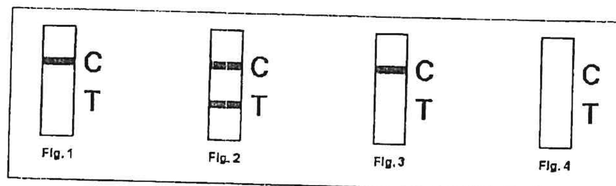
Il test risulta positivamente, fino ad una concentrazione di 2000 ug/ml nel mezzo di trasporto.

Raccolta campioni

Attenzione a non rompere la punta rossa del tubo di raccolta. Svitare il tappo rosso del tubo, tenendolo all'insù e assicurandosi di non rovesciare il mezzo di trasporto. Raccogliere i campioni del presunto sangue da varie parti con uno strumento idoneo. Immergere il campione nel mezzo di trasporto contenuto nel tubo e agitare gentilmente. Avvitare bene il tappo. Il campione è stabile a temperatura ambiente deve essere esaminato entro e non oltre una settimana da quando viene raccolto.

Procedura d'Esame e Interpretazione

1. Agitare il tubo prima dell'uso
2. Rimuovere il test dall'involucro e posizionarlo su di una superficie piatta.
3. Coprire il tappo rosso con un fazzoletto per evitare la dispersione. Tenere il tubo verticalmente e romperne la punta.
4. Distribuire esattamente **3 gocce** intere nel pozzetto campionario (S) nell'estremità inferiore del test.
5. Attendere 5 minuti prima di leggere il risultato, i risultati negativi vanno confermati dopo 10 minuti.



Negativi: (Fig.1)
Solo la linea di controllo rossa (C) appare nella parte superiore della finestra rettangolare riportante l'esito, indicando che il test sono eseguiti correttamente ed i reagenti hanno funzionato correttamente.

Positivo:(Fig. 2 e 3)
Una seconda riga rossa (riga-test T) appare nella parte inferiore della finestra rettangolare riportante l'esito, indicando un risultato positivo all'emoglobina nel campione. Se c'è una linea debole indica comunque un risultato positivo.

Invalido:(Fig.4)
Non appare nessuna riga di controllo . Ripetere l'esame con un nuovo test, seguendo le istruzioni attentamente.

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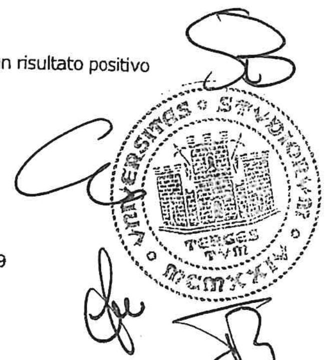


Prova 2

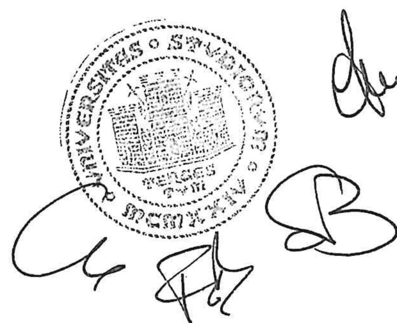
Descrivere come allestirebbe una reazione di PCR con il kit PowerPlex Fusion dei seguenti tre campioni di DNA, motivandone la logica.

Si tratta di tre campioni estratti da altrettanti tamponi salivari che sono stati spediti per posta a temperatura ambiente (durata della spedizione: 5 giorni).

I tre campioni di DNA sono stati risospesi in H_2O e quantificati sia con il Nanodrop (spettrofotometria UV; LOQ= 10-500 ng/µl; LOD= 1,5 ng/ µl) che con il kit Quantifiler (kit di quantitative PCR con LOQ= 0,023- 50,0 ng/µl; LOD= 0,001 ng/ µl).

Campione	Nanodrop		Quantifiler
	ng/µl	260/280	ng/µl
A	66,0	1,84	5,384
B	41,5	1,91	2,487
C	22,0	1,82	1,257

Le caratteristiche del kit PowerPlex Fusion sono indicate nell'**allegato 1** e si specifica che il controllo (2800M Control DNA) è fornito dalla Ditta alla concentrazione di 10 ng/µl.



3.B. Spectral Calibration

Proper spectral calibration is critical to evaluate multicolor systems with the ABI PRISM® 3100 and 3100-*Avant* Genetic Analyzers and Applied Biosystems® 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers. A matrix must be generated for each individual instrument.

For protocols and additional information about spectral calibration on these instruments, see the *PowerPlex® 5C Matrix Standard Technical Manual* #TMD049. This manual is available online at: **www.promega.com/protocols/**

4. Protocols for DNA Amplification Using the PowerPlex® Fusion System

The PowerPlex® Fusion System was developed for amplification of extracted DNA and direct-amplification samples. Slight protocol variations are recommended for optimal performance with each template source. Protocols for amplification in a 25µl reaction volume using extracted DNA (Section 4.A), lytic and nonlytic storage card punches (Section 4.B) and swabs (Section 4.C) are included in the following amplification sections. Protocols for amplification in a 12.5µl reaction volume using lytic and nonlytic storage card punches and swabs are included in Sections 9.C and 9.D, respectively.

The PowerPlex® Fusion System is compatible with the GeneAmp® PCR System 9700 with a gold-plated silver or silver sample block, the Veriti® 96-Well Thermal Cycler and the ProFlex® PCR System.

Note: It may be possible to use thermal cyclers other than those listed in this technical manual. Use of thermal cyclers not listed here may require optimization of cycling conditions and validation in your laboratory. Use of thermal cyclers with an aluminum block is **not** recommended with the PowerPlex® Fusion System.

The use of gloves and aerosol-resistant pipette tips is highly recommended to prevent cross-contamination. Keep all pre-amplification and post-amplification reagents in separate rooms. Prepare amplification reactions in a room dedicated for reaction setup. Use equipment and supplies dedicated for amplification setup.

Meticulous care must be taken to ensure successful amplification. A guide to amplification troubleshooting is provided in Section 7.

4.A. Amplification of Extracted DNA in a 25µl Reaction Volume

The following protocol has been routinely used to amplify 0.25–0.5ng of template DNA in a 25µl reaction volume using the PowerPlex® Fusion System.

Materials to Be Supplied by the User

- GeneAmp® PCR System 9700 with a gold-plated silver or silver sample block, Veriti® 96-Well Thermal Cycler or ProFlex® PCR System (Applied Biosystems)
- centrifuge compatible with 96-well plates or reaction tubes
- MicroAmp® optical 96-well reaction plate or 0.2ml MicroAmp® reaction tubes (Applied Biosystems)
- aerosol-resistant pipette tips

Amplification Setup

1. Thaw the PowerPlex® Fusion 5X Master Mix, PowerPlex® Fusion 5X Primer Pair Mix and Water, Amplification Grade, completely.

   Note: Centrifuge tubes briefly to bring contents to the bottom, and then vortex reagents for 15 seconds before each use. Do not centrifuge the 5X Primer Pair Mix or 5X Master Mix after vortexing, as this may cause the reagents to be concentrated at the bottom of the tube.

2. Determine the number of reactions to be set up. This should include positive and negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does consume a small amount of each reagent, it ensures that you will have enough PCR amplification mix for all samples. It also ensures that each reaction contains the same PCR amplification mix.

3. Use a clean plate for reaction assembly, and label it appropriately. Alternatively, determine the number of clean, 0.2ml reaction tubes required, and label them appropriately.

4. Add the final volume of each reagent listed in Table 1 to a clean tube.

Table 1. PCR Amplification Mix for Amplification of Extracted DNA.

PCR Amplification Mix Component[1]	Volume Per Reaction	×	Number of Reactions	=	Final Volume
Water, Amplification Grade	to a final volume of 25µl	×		=	
PowerPlex® Fusion 5X Master Mix	5µl	×		=	
PowerPlex® Fusion 5X Primer Pair Mix	5µl	×		=	
template DNA (0.25–0.5ng)[2,3,4]	up to 15µl				
total reaction volume	**25µl**				

[1]Add Water, Amplification Grade, to the tube first, and then add PowerPlex® Fusion 5X Master Mix and PowerPlex® Fusion 5X Primer Pair Mix. The template DNA will be added at Step 6.

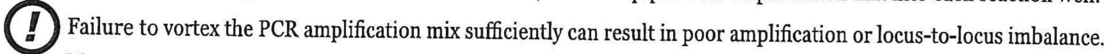
[2]Store DNA templates in TE^{-4} buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA) or TE^{-4} buffer with 20µg/ml glycogen. If the DNA template is stored in TE buffer that is not pH 8.0 or contains a higher EDTA concentration, the volume of DNA added should not exceed 20% of the final reaction volume. PCR amplification efficiency and quality can be greatly altered by changes in pH (due to added Tris-HCl), available magnesium concentration (due to chelation by EDTA) or other PCR inhibitors, which may be present at low concentrations depending on the source of the template DNA and the extraction procedure used.

[3]Apparent DNA concentrations can differ, depending on the DNA quantification method used (13). We strongly recommend that you perform experiments to determine the optimal DNA amount based on your DNA quantification method.

[4]The PowerPlex® Fusion System was optimized and balanced for 0.25–0.5ng of DNA template. The amount of DNA template used in your laboratory should be based on the results of your internal validation and may be different.

4.A. Amplification of Extracted DNA in a 25µl Reaction Volume (continued)

5. Vortex the PCR amplification mix for 5–10 seconds, and then pipet PCR amplification mix into each reaction well.



Failure to vortex the PCR amplification mix sufficiently can result in poor amplification or locus-to-locus imbalance.

Note: Do not store the PCR amplification mix for a prolonged period. Add the mix to the wells of the reaction plate as soon as the mix is prepared. Add DNA as soon as possible to each well and follow immediately by thermal cycling.

6. Add template DNA for each sample to the respective well containing PCR amplification mix.

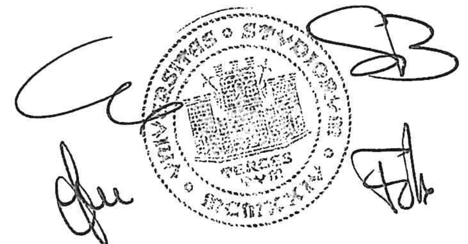
   Note: The PowerPlex® Fusion System is optimized and balanced using 0.25–0.5ng of DNA template. The amount of DNA template used in your laboratory should be based on the results of your internal validation and may be different.

7. For the positive amplification control, vortex the tube of 2800M Control DNA, and then dilute an aliquot to 0.5ng in the desired template DNA volume. Add 0.5ng of diluted DNA to a reaction well containing PCR amplification mix.

8. For the negative amplification control, pipet Water, Amplification Grade, or TE^{-4} buffer instead of template DNA into a reaction well containing PCR amplification mix.

9. Seal or cap the plate, or close the tubes.

   Optional: Briefly centrifuge the plate to bring contents to the bottom of the wells and remove any air bubbles.



Thermal Cycling

Amplification and detection instrumentation may vary. You may need to optimize protocols including the amount of template DNA, cycle number, injection conditions and loading volume for your laboratory instrumentation. Testing at Promega shows that 30 cycles works well for 0.5ng of purified DNA template. In-house validation should be performed.

1. Place the reaction plate or tubes in the thermal cycler.
2. Select and run the recommended protocol, which is provided in Figure 1.

   Notes:

   1. When using the ProFlex® PCR System, use the 9700 Simulation Mode.
   2. When using the Veriti® 96-Well Thermal Cycler, set the ramping rate to 100%.
   3. When using the GeneAmp® PCR System 9700, the program must be run with Max Mode as the ramp speed. This requires a gold-plated silver or silver sample block. The ramp speed is set after the thermal cycling run is started. When the 'Select Method Options' screen appears, select "Max" for the ramp speed and enter the reaction volume.

Thermal Cycling Protocol
96°C for 1 minute, then:
94°C for 10 seconds 59°C for 1 minute 72°C for 30 seconds for 30 cycles, then:
60°C for 10 minutes
4°C soak

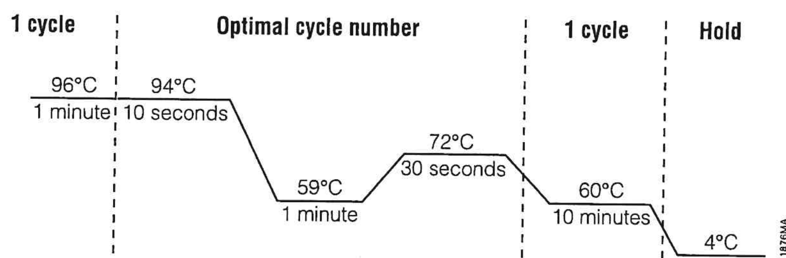


Figure 1. Thermal cycling protocol for the GeneAmp® PCR System 9700, Veriti® 96-Well Thermal Cycler and ProFlex® PCR System.

3. After completion of the thermal cycling protocol, proceed with fragment analysis or store amplified samples at −20°C in a light-protected box.

   Note: Long-term storage of amplified samples at 4°C or higher may produce artifacts.